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Title: FoxO inhibits juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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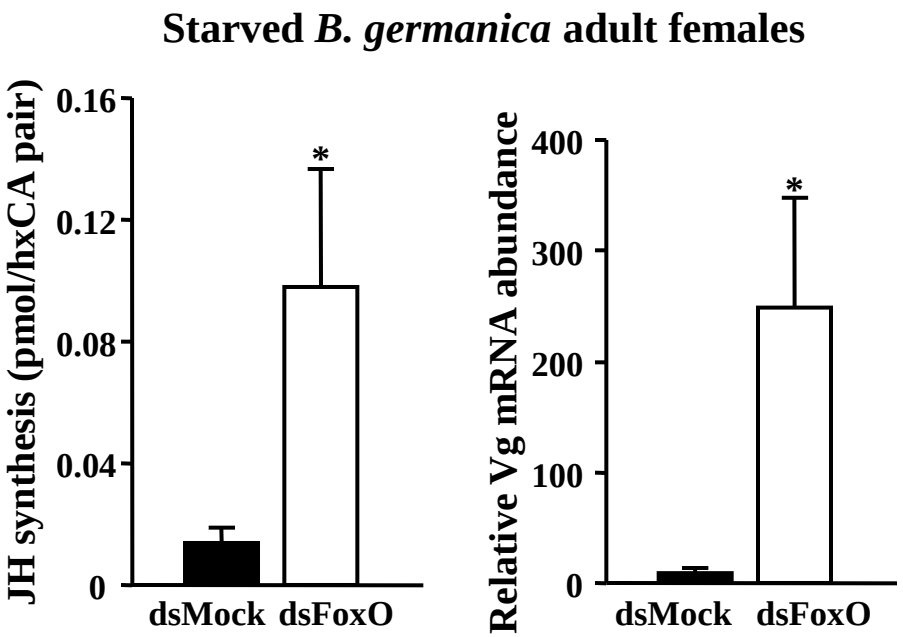
Abstract: The transcription factor Forkhead-box O (FoxO) is the main transcriptional effector of the Insulin Receptor/Phosphatidylinositol 3-kinase (InR/PI3K) pathway. In a situation of nutrient restriction, the pathway is inactive and FoxO translocates to the nucleus to exert its transcriptional action. In starved females of the cockroach Blattella germanica, the reproductive processes, and in particular the synthesis of juvenile hormone in the corpora allata and that of vitellogenin in the fat body, are arrested. In the present report we examine the possible role of FoxO in the transduction of the nutritional signals to these reproductive events. We first cloned FoxO cDNA from B. germanica (BgFoxO), and showed that its expression is not nutritionally regulated. BgFoxO knockdown using systemic RNAi in vivo in starved females elicited an increase of juvenile hormone biosynthesis, although without modifying mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase or methyl farnesoate epoxidase (CYP15A1) in corpora allata. In addition, BgFoxO RNAi treatment produced a remarkable increase of vitellogenin mRNA levels in fat body and of vitellogenin protein in the haemolymph. Our results indicate that BgFoxO plays an inhibitory role on juvenile hormone biosynthesis and vitellogenin production in a situation of nutrient shortage.

*Graphical Abstract



Highlights

-Previous studies demonstrated that starvation inhibits juvenile hormone biosynthesis and vitellogenin production.

-Expression pattern indicates that *B. germanica* FoxO is not transcriptionally regulated during starvation.

-BgFoxO RNAi treatment in starved females increases juvenile hormone biosynthesis and vitellogenin production.

-Results suggest that BgFoxO inhibits juvenile hormone biosynthesis and vitellogenin production during nutrient shortage.

FoxO inhibits juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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Abstract

The transcription factor Forkhead-box O (FoxO) is the main transcriptional effector of the Insulin Receptor/Phosphatidylinositol 3-kinase (InR/PI3K) pathway. In a situation of nutrient restriction, the pathway is inactive and FoxO translocates to the nucleus to exert its transcriptional action. In starved females of the cockroach *Blattella germanica*, the reproductive processes, and in particular the synthesis of juvenile hormone in the corpora allata and that of vitellogenin in the fat body, are arrested. In the present report we examine the possible role of FoxO in the transduction of the nutritional signals to these reproductive events. We first cloned FoxO cDNA from *B. germanica* (BgFoxO), and showed that its expression is not nutritionally regulated. BgFoxO knockdown using systemic RNAi *in vivo* in starved females elicited an increase of juvenile hormone biosynthesis, although without modifying mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase or methyl farnesoate epoxidase (CYP15A1) in corpora allata. In addition, BgFoxO RNAi treatment produced a remarkable increase of vitellogenin mRNA levels in fat body and of vitellogenin protein in the haemolymph. Our results indicate that BgFoxO plays an inhibitory role on juvenile hormone biosynthesis and vitellogenin production in a situation of nutrient shortage.

Keywords: FoxO, insulin, juvenile hormone, vitellogenin, *Blattella germanica*, nutritional signalling

1. Introduction

Insect vitellogenesis involves coordinated activities between corpora allata (CA), fat body and ovaries. In many insect species, juvenile hormone (JH) synthesized and secreted by the CA activates vitellogenin (Vg) production in the fat body and its incorporation into the maturing oocytes (Belles, 2005). The regulation of JH synthesis and vitellogenin production includes different factors as neuropeptides, biogenic amines and nutritional signals, among others (Rauschenbach et al., 2004; Belles, 2005; Maestro et al., 2009). In the adult females of the cockroach *Blattella germanica*, JH biosynthesis shows a cyclic pattern, with a steady increase after the adult moult and a sudden decrease at oviposition time, remaining at low levels during the period of ootheca transport, and initiating a new gonadotrophic cycle after the eclosion of the nymphs from the ootheca (Maestro et al., 1994).

As a typical anautogenous species, the ingestion of a meal is a necessary requirement for initiating vitellogenesis in *B. germanica*. Thus, *B. germanica* starved females produce very low levels of JH and the Vg production is extremely reduced, whereas re-feeding triggers the vitellogenic processes (Osorio et al., 1997; Maestro et al., 2009). One of the signalling pathways involved in transducing the nutritional signals is the "target of rapamycine" (TOR) pathway. Knock-down of *TOR* expression produces a severe inhibition of JH production in adult females of *B. germanica* (Maestro et al., 2009). In addition, these females show a reduction of Vg mRNA in the fat body, not only derived from the reduced JH levels, but also due to a lack of a positive effect of TOR on *Vg* transcription (Maestro et al., 2009).

Besides TOR, other mechanisms have been related to nutritional signalling, particularly the Insulin Receptor/Phosphatidylinositol 3-kinase (InR/PI3K) pathway. Insect insulin-related peptides (IRP), analogously to vertebrate insulin, are secreted in response to different factors, especially high nutritional levels (Masumura et al., 2000; Ikeya et al., 2002; Geminard et al., 2009). Insulin receptor-mediated activation of PI3K increases the production of phosphatidylinositol trisphosphate (PIP_3) that, acting as a second messenger, recruits Akt to the plasma membrane. Once in the plasma membrane, Akt is phosphorylated and, in turn, it phosphorylates a number of downstream targets that ultimately carry out pathway effects (Puig et al., 2003; Baker & Thummel, 2007). One of the proteins phosphorylated by Akt is the transcription factor Forkhead-box, class O (FoxO), which has been revealed as the most relevant agent for

the transcriptional activities of the InR/PI3K pathway (Murphy et al., 2003; Gershman et al., 2007).

FoxO proteins are a subgroup of the Forkhead-box family of transcription factors. This family comprises a large and diverse group of proteins characterized by a conserved DNA-binding domain (the Forkhead-box or Fox), classified from FoxA to FoxS on the basis of sequence similarity. Members of the class "O" share the characteristic of being regulated by the InR/PI3K signalling pathway by Akt phosphorylation at three conserved residues (Barthel et al., 2005; Greer & Brunet, 2005). This phosphorylation leads to the export of FoxO protein from the nucleus to the cytoplasm (Greer & Brunet, 2005; Baker & Thummel, 2007). Feeding condition, thus, maintains FoxO inactive in the cytoplasm, whereas starvation promotes its transcriptional activities upon nutritionally regulated genes in the nucleus.

In *B. germanica*, the mRNA levels of some genes related to reproductive processes are modified in starvation. mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1 and -2 and HMG-CoA reductase, enzymes of the mevalonate pathway but also involved in JH synthesis in the CA, are inhibited during starvation (Maestro et al., 2009). In addition, *Vg* expression in fat body is also reduced in starved females (Maestro et al., 2009). In this study, we analyzed the function of FoxO in *B. germanica* adult females, and in particular its relationship with the processes related to reproduction.

2. Materials and Methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared with dog chow and water, in the dark at 30 ± 1°C and 60-70% relative humidity. For the study of gene expression levels during the first gonadotrophic cycle, virgin females were used. For the RNAi experiments, we induced a second gonadotrophic cycle by removing the ootheca at the twelfth day of its transport period. For the starvation experiments, animals received only water after the imaginal moult or after the induction of the second gonadotrophic cycle. Dissections of CA and abdominal fat body were carried out on carbon dioxide-anesthetized specimens.

2.2. Cloning of BgFoxO cDNA

Degenerated primers based on conserved regions of insect and vertebrate FoxO sequences were used to obtain a *B. germanica* homologue cDNA fragment by RT-PCR. The first PCR amplification was carried out using cDNA generated by reverse transcription from RNA of UM-BGE-1 cells (derived from early embryos of *B. germanica*) as a template. Primers can be found as supplementary data, Table 1. We amplified a 101 bp fragment, which was subcloned into the pSTBlue-1 vector (Novagen; 70596) and sequenced. This was followed by 3´-RACE and several 5´-RACEs (5´- and 3´-RACE System Version 2.0; Invitrogen) using different specific primers to complete the sequence. We used again cDNA from UM-BGE-1 cells as a template. PCR products were subcloned into the pSTBlue-1 vector and sequenced in both directions.

2.3. Phylogenetic analyses

We used the sequences from the following insects: *Drosophila melanogaster* (GenBank™ Accession Number: NP_996204), *Aedes aegypti* (ABK76646), *Anopheles gambiae* (CAD27476), *Nasonia vitripennis* (XP_001607658), *Apis mellifera* (XP_001122804), *Tribolium castaneum* (XP_975200), *Pediculus humanus* (XP_002425837) and *Acyrthosiphon pisum* (XP_001944722); the Nematoda: *Caenorhabditis elegans* (AAC47803); the Echinodermata: *Strongylocentrotus purpuratus* (ABB89484); and the Chordata: *Ciona intestinalis* (NP_001071717), *Gallus gallus* FoxO 3 (XP_001234496), *Xenopus laevis* FoxO 3 (AAI70411), *Homo sapiens* FoxO 1 (AAH70065), *H. sapiens* FoxO 3 (NP_001446) and *H. sapiens* FoxO 4 (NP_005929). The tree was rooted in the divergence between protostomes and deuterostomes. Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana, 2000). The obtained alignment was analyzed with the PHYML 3.0 program (Guindon & Gascuel, 2003), based on the maximum-likelihood principle. Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates.

2.3. RNA extraction, cDNA synthesis and Real-time PCR analyses

The CA and fat body expression levels of the different studied genes were analyzed using real-time PCR. cDNA was synthesized from total RNA as previously described (Maestro & Belles, 2006). An amount of 0.5 µg of total RNA was used in the case of fat bodies, whereas in the case of CA, the whole RNA from one pair of glands was used. The absence of genomic contamination was checked using a control without reverse transcription.

cDNA levels were quantified by using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad) as previously described (Maestro et al., 2010). Primer sequences to amplify BgFoxO, HMG-CoA synthase-1 and -2, HMG-CoA reductase, methyl farnesoate epoxidase (CYP15A1), Vg and Actin 5C (used as a reference), are reported as supplementary data, Table 1. Total reaction volume was 20 µl. All reactions were run in duplicate or triplicate. The program used to amplify the reaction was as follows: (i) 95˚C for 3 min; (ii) 95˚C for 10 sec; (iii) 60˚C for 1 min; and (iv) repeat to step ii for 50 cycles. Real-time data were collected by iQ5 optical system software v. 2.0 (Bio-Rad).

2.4. RNA Interference

Systemic RNAi *in vivo* in females of *B. germanica* was performed as previously described (Maestro et al., 2009). Two different fragments, a 570-bp dsRNA fragment (dsFoxO) encompassing most of the Forkhead-box DNA binding domain and the 3'-contiguous sequence, and a 298-bp fragment (dsFoxO II) encompassing the protein C-terminus and part of the 3' non-coding region, spanning positions 868 to 1437 and 1918 to 2215, respectively, of the BgFoxO cDNA, were used to generate two different dsRNA (Fig. 1B). As a control, a heterologous 307-bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsMock) was used (Maestro et al., 2011). To be confident that the treatment produced a sufficient BgFoxO reduction, we performed long time treatments. They consist in the injection of 2 µg of the dsRNA fragment into the abdomen of females in the first day of ootheca transport. Twelve days later, we removed the ootheca, triggering the beginning of a second gonadotrophic cycle in all respects similar to the first one. Dissections were carried out 5 days later. In addition, using this experimental model we avoid the possible developmental phenotypes caused by RNAi treatment at the nymphal stages and only concentrate in the

effects of BgFoxO RNAi treatment in the adult. BgFoxO, HMG-CoA synthase-1 and -2, HMG-CoA reductase, CYP15A1, Vg, and Actin 5C (used as a reference) mRNA levels were determined by RT-qPCR.

2.5. Quantification of juvenile hormone synthesis

JH III biosynthesis by CA incubated *in vitro* was quantified as previously reported by (Piulachs & Couillaud, 1992). Essentially, individual pairs of CA were incubated in 100 µl of 199 medium (Sigma) containing L-methionine (0.1 nM), Hank's salts, Hepes buffer (20 mM) plus Ficoll (20 mg/ml), to which L-[^{3}H-methyl]methionine (Amersham Biosciences) had been added, to achieve a final specific activity of 7.4 GBq/mmol. CA were incubated for 3 h, after which JH III was quantified in the medium plus homogenized glands.

2.6. Haemolymph collection and Western blot analysis

Haemolymph samples were collected in a microcapillary pipette, after cutting off the metathoracic legs, diluted in NaCl 0.4M plus 4% Protease Inhibitor (Sigma), fast frozen in liquid nitrogen and stored at –80ºC. Samples were centrifuged at 10,000 x g for 10 min at 4ºC before analysis. Similar volumes of individual haemolymph samples were separated using 7.5 % polyacrilamide gel electrophoresis. Western blot analyses were performed using an anti-*B. germanica* vitellin antiserum as described by (Martin et al., 1995), which also detects vitellogenin. As secondary antibody, a HRP-conjugated anti-rabbit immunoglobulin (PIERCE) was used. The signal was visualized using Thermo Scientific Super Signal West Femto Substrate (PIERCE).

3. Results

3.1. Cloning of BgFoxO cDNA, sequence comparison and phylogenetic analysis

Using degenerate primers and cDNA from the *B. germanica* UM-BGE 1 cells as template, a 101-bp fragment of a presumed FoxO homologue of *B. germanica* (BgFoxO) was obtained. To complete the cDNA sequence, we followed 3'-RACE and 5'-RACE methods, and we obtained a sequence of 2282 bp (GenBank accession

number: HE648216), which encoded a protein of 523 amino acids. The putative start codon was preceded by an in-frame stop codon and the final stop codon was followed by a poly(A) sequence, suggesting that it was a full-length ORF. BLAST database search indicated that the protein was a *B. germanica* homologue of FoxO. The aminoacid sequence shows 58% identity when compared with *T. castaneum* FoxO, 43% with *D. melanogaster* FoxO, and 30% with *H. sapiens* FoxO3. These percentages increase when we compare only the Fox DNA-binding domain, reaching 96% when compared with *T. castaneum*, 91% with *D. melanogaster* and 77% with *H. sapiens* FoxO3. BgFoxO shows the typical organization of FoxO proteins, with the 5-aminoacid insertion between helices 2 and 3, which is characteristic of FoxO and is lacking in the other Forkhead-box-related proteins (Puig et al., 2003; Hansen et al., 2007). In addition, it shows the three FoxO characteristic Akt phosphorylation motifs in T_{23}, S_{176} and S_{243} (Greer & Brunet, 2005).

Maximum-likehood analysis including the BgFoxO sequence and other representative FoxO sequences available in databases, gave the tree shown in Fig. 1A. With the root of the tree placed in the divergence between protostomes and deuterostomes, the topology does not differ from the current phylogeny of the included species, and indicates that the *B. germanica* sequence corresponds to a FoxO protein. In addition, the tree also indicates that the diversification of FoxO proteins present in Vertebrata derives from duplications occurring in this particular lineage.

3.2. Expression patterns of BgFoxO

mRNA levels of BgFoxO were studied in CA and fat bodies of adult females throughout the first gonadotrophic cycle and under starvation. BgFoxO mRNA levels in the CA were practically constant throughout the gonadotrophic cycle (Fig. 2A), whereas fat body levels remained constant until mid vitellogenesis and increased thereafter up to oviposition time (Fig. 2A). For the analysis of FoxO mRNA levels in starved vs. fed adult females, we used 5-day old *B. germanica* females that had been maintained only with water since the imaginal moult. Results showed that there are not significant differences in mRNA BgFoxO levels between fed and starved animals neither in CA nor in fat body (Fig. 2B).

3.3. Effects of BgFoxO RNAi

To study the function of BgFoxO, its expression was lowered using systemic RNAi. In fed animals, dsFoxO treatment induced a significant 62% decrease of mRNA BgFoxO in CA (Fig. 3A), although this was not reflected on JH synthesis, which showed similar values between dsMock and dsFoxO females (Fig. 3B). Concerning fat bodies, dsFoxO treatment produced a 89% of mRNA BgFoxO reduction and a significant 76% inhibition of mRNA Vg levels, concomitant with a reduction in length of the growing oocytes (Fig. 3C-E). Whereas a 100% of the dsMock-treated females could form a proper ootheca, none of the dsFoxO-treated females succeeded in forming a viable ootheca.

In the case of starved females, a significant 67% reduction in CA mRNA BgFoxO levels elicited by the RNAi treatment, induced a 7-fold increase in JH synthesis (Fig. 4 A, B). This increase in JH synthesis was not matched by an increase in mRNA levels of the enzymes HMG-CoA synthase-1 and 2 and HMG-CoA reductase, which showed similar levels between dsMock and dsFoxO-treated animals (Fig. 4C).

Analysing other enzymes of the JH biosynthetic pathway, we demonstrated that starvation induced an 82% inhibition in mRNA levels of CYP15A1 (Fig. 2C), a methyl farnesoate epoxidase that has been recently cloned from *B. germanica* CA (Maestro et al., 2010). Nevertheless, and as it occurs for HMG-CoA synthases and reductase, BgFoxO RNAi treatment did not modify CYP15A1 mRNA levels (Fig. 4C).

Concerning the fat body of starved females, dsFoxO treatment induced a 77% reduction of BgFoxO mRNA levels (Fig. 5A), together with a ca. 25-fold stimulation of Vg mRNA levels (Fig. 5B) and a remarkable increase of Vg protein concentration in the haemolymph, measured by Western blot (Fig. 5C).

Equivalent RNAi experiments using a dsRNA based on a BgFoxO cDNA fragment (dsFoxO II, Fig. 1B) which does not overlap with the one used in the previous experiments, resulted in similar phenotypes. Then, we found an 82% reduction in fat body BgFoxO mRNA, concomitant with a 22-fold increase of Vg mRNA levels in starved dsFoxO II compared to dsMock-treated females (supplementary data, Fig. 1). In addition we quantified a 4-fold increase in JH synthesis in dsFoxO II compared to ds Mock females (supplementary data, Fig. 1). The similarity of results between dsFoxO and dsFoxO II experiments indicate that these effects are specific for BgFoxO knockdown.

4. Discussion

We have cloned and characterized BgFoxO as the FoxO homologue in the German cockroach, *B. germanica*. BgFoxO shows the characteristic organization of FoxO proteins, with a Forkhead-box DNA-binding domain with the distinguishing traits of the class O. The sequence also shows the three putative Akt Ser or Thr phosphorylation motifs (RXRXXS/T) at the characteristic FoxO positions (Greer & Brunet, 2005). Thus, sequence of the cloned cDNA indicates that it is the *B. germanica* homologue of FoxO at that it could be nutritionally regulated through the InR/PI3K pathway.

BgFoxO mRNA levels in CA remain constant through the first gonadotrophic cycle. Conversely, BgFoxO mRNA fat body levels are constant until mid vitellogenesis and increases later on. In the mosquito *Ae. aegypti FoxO* expression in the fat body inversely correlates with *Vg* expression (Hansen et al., 2007). This is not the case in *B. germanica*, where *Vg* expression shows a peak at day 5 (Martin et al., 1998), when BgFoxO mRNA levels are increasing. It is also notable that relative BgFoxO mRNA levels in fat body are between four and ten times higher than those of CA. Further work is needed for understanding these differences in profile and levels between an endocrine organ (CA) and a mainly metabolic tissue (fat body).

BgFoxO mRNA levels were also analyzed in CA and fat bodies from fed and starved 5 day-old adult *B. germanica* females. Results showed no differences between BgFoxO mRNA levels neither on CA nor in fat bodies, which indicates that nutritional status does not regulate *BgFoxO* expression and suggests that BgFoxO activity is regulated at post-transcriptional level. Regulation of FoxO activity through phosphorylation, acetylation or ubiquitination has been reported for FoxO proteins. These modifications alter mostly FoxO subcellular localization (nuclear or cytoplasmic), but can also affect FoxO degradation, DNA-binding ability, transcriptional activity or protein-protein interactions (Barthel et al., 2005; Greer & Brunet, 2005).

BgFoxO RNAi treatment in fed specimens produced a 76% reduction of Vg mRNA levels in fat body, concomitant with a reduction of oocyte length, without a significant inhibition of JH synthesis. Similarly, FoxO knockdown produces reduction of Vg2 mRNA levels in fed females of the beetle *T. castaneum* (Parthasarathy & Palli, 2011). InR/PI3K pathway forms the central component of FoxO regulation by acting on

Akt, and then, in fed animals BgFoxO is supposed to be Akt-phosphorylated at their putitative regulation sites, keeping the protein inactive at the cytoplasm (Barthel et al., 2005; Greer & Brunet, 2005; Baker & Thummel, 2007). Nevertheless, other regulatory pathways can act upon FoxO activity. For example, oxidative stress causes FoxO to translocate to the nucleus where it stimulates the expression of target genes that encode proteins involved in oxidative stress protection. This response can be mediated by the Jun N-terminal kinase (JNK) signalling pathway (Wang et al., 2005; Lee et al., 2009). Thus, BgFoxO knockdown in fed animals would disturb the handling of an increase oxidative stress situation and this could be reflected by a final effect on *Vg* expression.

In the case of starved *B. germanica* females, BgFoxO RNAi treatment induced a significant 7-fold increase in JH synthesis. It has already been reported that starvation reduces *B. germanica* females JH synthesis (Maestro et al., 2009). The increase in JH synthesis in dsFoxO compared with dsMock-treated starved females suggests that BgFoxO activity is involved in the inhibition of JH synthesis caused by starvation. A direct involvement of the InR/PI3K pathway on JH biosynthesis, and then a possible involvement of FoxO, has been reported for *D. melanogaster*. *InR* and *chico* fly mutants have reduced JH synthesis (Tatar et al., 2001; Tu et al., 2005). However, another report maintains that *chico* mutants synthesize JH at normal levels (Richard et al., 2005).

Even so, our results show that dsFoxO treatment in starved females do not fully restore JH biosynthesis levels observed in fed females. In *B. germanica*, it has been reported that the TOR pathway is related to the activation of JH biosynthesis (Maestro et al., 2009). The TOR pathway will be inactive in starved females and this may explain why JH biosynthesis was not completely recovered although we had reduced BgFoxO levels.

The expression of different enzymes of the JH biosynthetic pathway, HMG-CoA synthase-1 and -2, and HMG-CoA reductase and CYP15A1 is inhibited in the CA of *B. germanica* starved females (Maestro et al., 2009), and present results). The same results are observed when comparing fed and starved females in the second gonadotrophic cycle (results not shown). Nevertheless, the partial relief of JH biosynthesis inhibition observed in BgFoxO knockdown starved females do not derive from an increase in the expression of any of these enzymes. These results suggest that the inhibitory action of BgFoxO on JH biosynthesis observed in starved females should be located at other regulatory points as, for example, gene expression of other enzymes of the JH biosynthesis pathway or at post-transcriptional levels. One possible candidate could be

juvenile hormone acid methyl transferase (JHAMT), enzyme that catalyses the penultimate step of JH synthesis, and has been suggested to be a key enzyme for JH synthesis in the Eri Silkworm (Sheng et al., 2008). Nevertheless, ca. 25% reduction observed in *T. castaneum* and *Ae. aegyti* JHAMT mRNA levels under starvation or nutrition deficiency is not significant when compared to fed controls (Nouzova et al., 2011; Parthasarathy & Palli, 2011), and these would indicate that JHAMT is not a candidate to be regulated by FoxO in response to food restriction. The possible participation of FoxO in post-transcriptional regulation of some of the enzymes of the JH biosynthetic pathway deserves to be explored in the future, given that some of these enzymes are reported to be highly post-transcriptionally regulated (Feyereisen & Farnsworth, 1987; Casals et al., 1997).

BgFoxO RNAi treatment in *B. germanica* starved females produced a huge induction of *Vg* expression, which was also reflected in an increase of Vg in the haemolymph. These results suggest that BgFoxO is repressing *Vg* expression during starvation. An inhibitory effect of daf-16, the *C. elegans* FoxO homologue, on *Vg* expression has already been reported in this nematode (Murphy et al., 2003). In addition, a recent work showed that the expression of *Vg* genes in the beetle *T. castaneum* is under the control of FoxO regulated by the InR/PI3K pathway (Sheng et al., 2011). These authors also demonstrate that FoxO can bind to a FRE (FoxO-response element) present in *Vg2* promoter (Sheng et al., 2011), and suggest that in this beetle, JH regulates *Vg* gene expression by inducing the expression of an insulin-like peptide, which releases *Vg* expression from the inhibitory effect of FoxO (Sheng et al., 2011). In *B. germanica*, the proximal promoter region of the *Vg* gene also contains three putative FRE (M.D. Piulachs, unpublished results), which supports the idea of a direct FoxO regulation on *Vg* transcription.

Nevertheless, BgFoxO knockdown in *B. germanica* starved females did not restore Vg mRNA levels observed in fed females, which suggests that some other factors must be involved in the low levels of *Vg* expression during starvation. One of these factors can be the TOR pathway, which has been reported to regulate *Vg* expression in different insect models, including *B. germanica* (Hansen et al., 2004; Maestro et al., 2009; Parthasarathy & Palli, 2011). In the mosquito *Ae. aegypti*, *Vg* expression is regulated in a nutrient-dependent manner by a GATA transcription factor, whose translation is activated through the TOR pathway (Park et al., 2006). Again, the proximal promoter region of *B. germanica Vg* gene contains at least two putitative

GATA response elements (M.D. Piulachs, unpublished results), which might be involved in the reported TOR regulation of *Vg* expression.

In summary, our results indicate that BgFoxO knockdown in starved *B. germanica* females produces the activation of JH biosynthesis and of *Vg* gene expression, and suggest that BgFoxO inhibits these processes in a situation of nutrient shortage.

Acknowledgments

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FIGURE LEGENDS

Fig. 1. Phylogenetic relationships of *B. germanica* FoxO and BgFoxO domain organization. (A) The tree was constructed using maximum-likehood approach. Branch lengths are proportional to sequence divergence. The bar represents 0.2 substitutions per site. Bootstrap values (100 replicates) are shown in the nodes. The tree was rooted in the divergence between protostomes and deuterostomes. (B) Scheme of BgFoxO domain organization showing the regions used to generate the two dsRNAs (dsFoxO and dsFoxO II). The position of the three conserved putative Akt-phosphorylation sited is showed. The striped box indicates the DNA-binding domain.

Fig. 2. Expression patterns of BgFoxO mRNA in the CA and the fat body of *B. germanica* adult females. (A) BgFoxO mRNA levels during the first gonadotrophic cycle in CA (n=3) and fat body (n=3-4). (B and C) BgFoxO mRNA levels in CA (n=10-

12) and fat bodies (FB) (n=19-22) (B), and CYP15A1 mRNA levels in CA (n= 5-6) (C), from fed and starved 5-day old adult *B. germanica* females. Starved females received only water after the imaginal moult. Y-axis indicates copies per copy of Actin 5C. Results expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, ***P<0.0001).

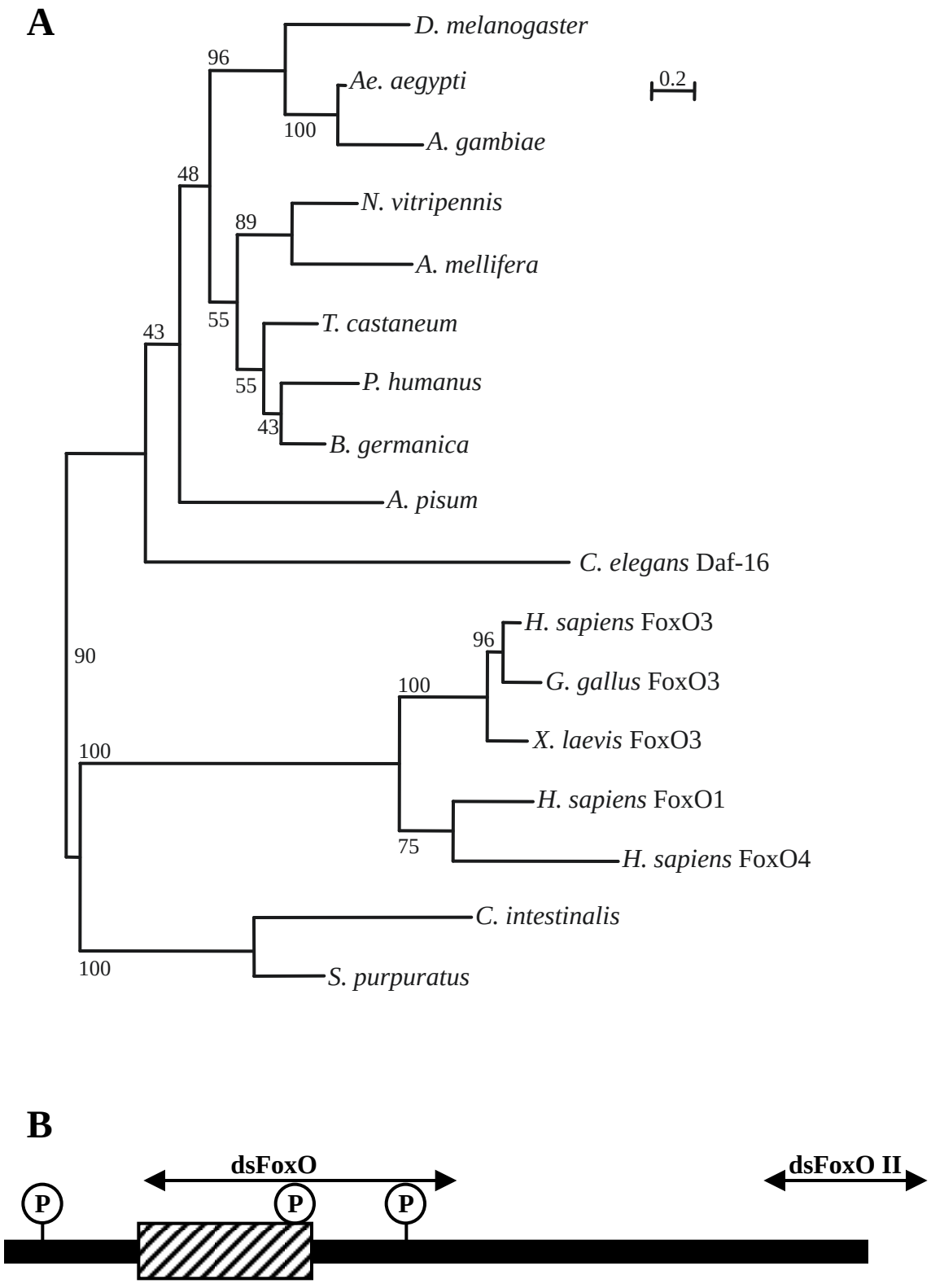
Fig. 3. Effect of BgFoxO RNAi on fed *B. germanica* females. dsRNA targeting BgFoxO (*dsFoxO*) or a non-specific dsRNA (*dsMock*) were administered in the first day of ootheca transport. The ootheca was removed 12 days later and dissections were performed at day 5 of the induced second cycle. Animals were continuously provided with food (dog chow) and water *ad libitum*. (A) BgFoxO mRNA levels in CA (n=9-10). (B) Rates of JH synthesis by CA incubated *in vitro* (n=9-11). (C) BgFoxO mRNA levels in fat bodies (n=8). (D) Vg mRNA levels (n=8). (F) Basal oocyte length (n=13-14). In (A), (C) and (D) Y-axis indicates copies per copy of Actin 5C. Results expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, **P<0.005; ***P<0.0001).

Fig. 4. Effect of BgFoxO RNAi on CA from starved *B. germanica* females. dsRNA targeting BgFoxO (*dsFoxO*) or a non-specific dsRNA (*dsMock*) were administered in the first day of ootheca transport. The ootheca was removed 12 days later and dissections were performed at day 5 of the induced second gonadotrophic cycle. Animals received only water after the induction of the second cycle. (A) BgFoxO mRNA levels (n=8-10). (B) Rates of JH synthesis by CA incubated *in vitro* (n=9-10). (C) mRNA levels of HMG-CoA synthase-1, -2, HMG-CoA reductase, and CYP15A1 (n=6-9). In (A) and (C) Y-axis indicates copies per copy of Actin 5C. Results expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, *P<0.05; **P<0.001).

Fig. 5. Effect of BgFoxO RNAi on fat bodies from starved *B. germanica* females. dsRNA targeting BgFoxO (*dsFoxO*) or a non-specific dsRNA (*dsMock*) were administrated in the first day of ootheca transport. The ootheca was removed 12 days later and dissections were performed at day 5 of the induced second gonadotrophic cycle. Animals received only water after the induction of the second cycle. (A) BgFoxO mRNA levels (n=9-11). (B) Vg mRNA levels (n=8-9). Y-axis indicates copies per copy

of Actin 5C. Results expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, **P*<0.05; ***P*<0.001). (C) Western blot determining haemolymph Vg protein levels. Blot shows the 160 kb band of Vg and is representative of 5 replicates.

Figure(s)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A. gambiae
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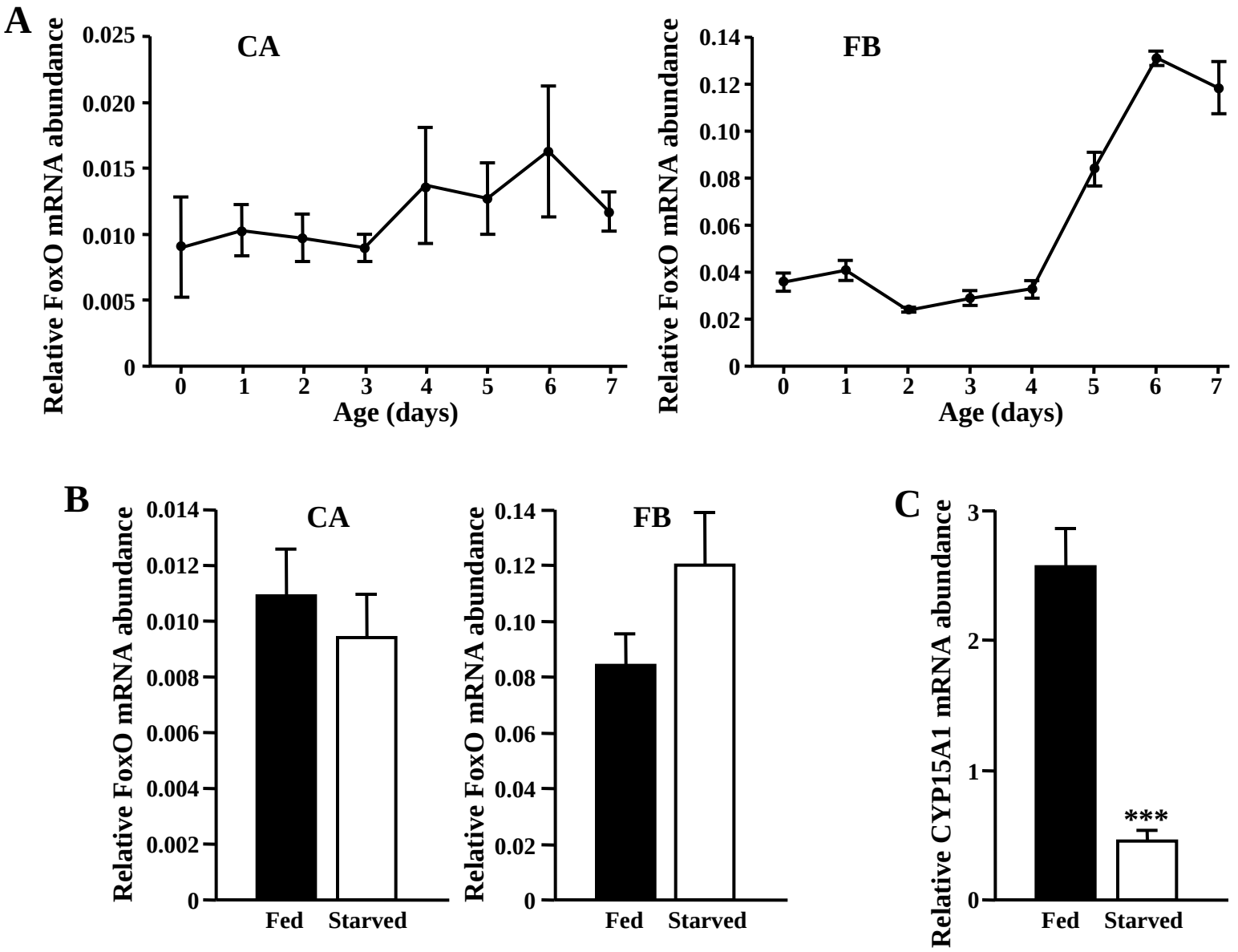
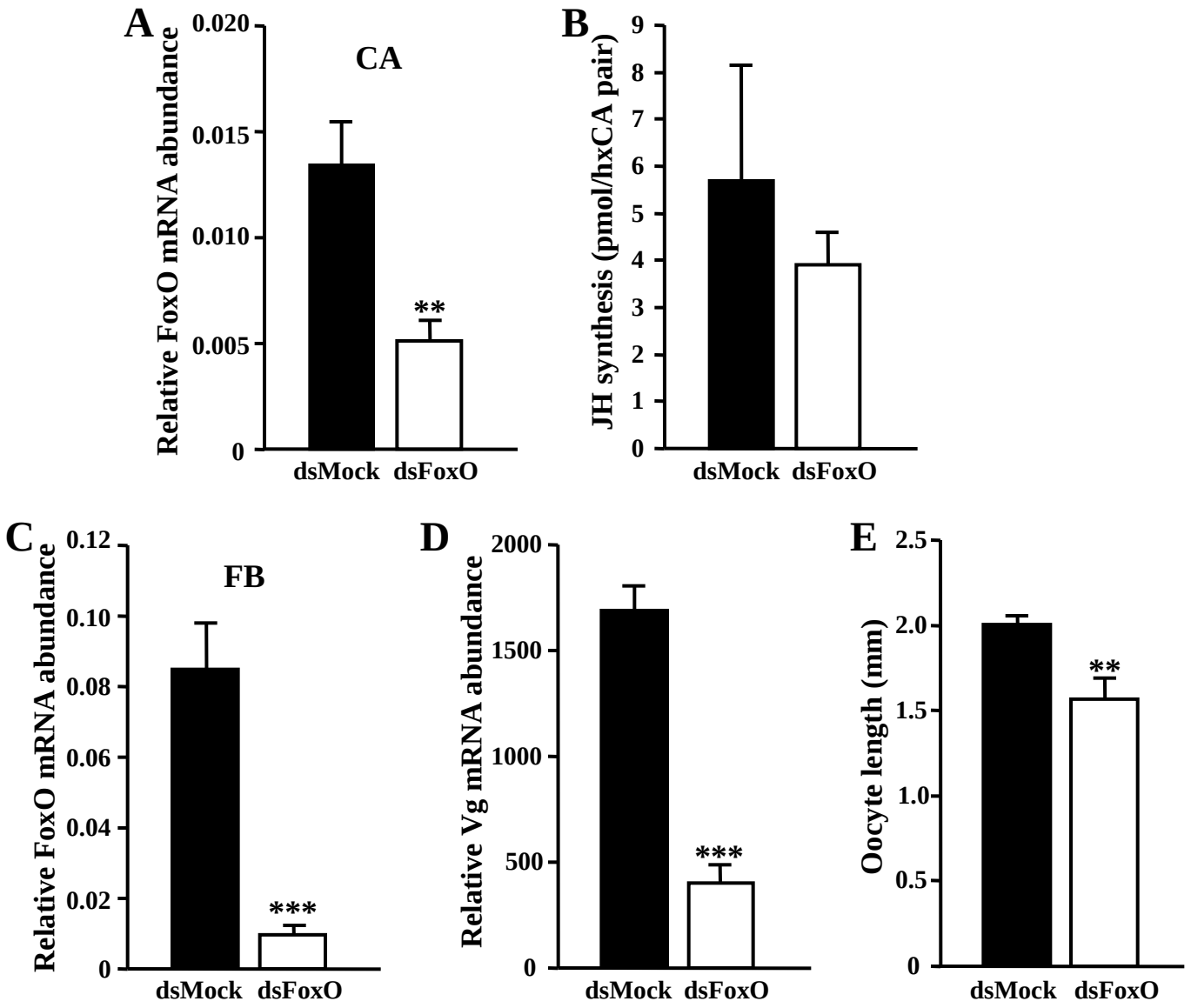
C. elegans Daf-16
H. sapiens FoxO3
G. gallus FoxO3
X. laevis FoxO3
H. sapiens FoxO1
H. sapiens FoxO4
C. intestinalis
S. purpuratus
0.2
96
100
48
89
55
43
55
43
90
96
100
100
75
100
B
dsFoxO
dsFoxO II
P
P
P

Figure(s)


A
CA
Relative FoxO mRNA abundance
0.025
0.020
0.015
0.010
0.005
0
0 1 2 3 4 5 6 7
Age (days)
FB
Relative FoxO mRNA abundance
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0 1 2 3 4 5 6 7
Age (days)
B
CA
Relative FoxO mRNA abundance
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
Fed Starved
FB
Relative FoxO mRNA abundance
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Fed Starved
C
Relative CYP15A1 mRNA abundance
3
2
1
0
***
Fed Starved

Figure(s)


A
CA
Relative FoxO mRNA abundance
0.020
0.015
0.010
0.005
0
**
dsMock
dsFoxO
B
JH synthesis (pmol/hxCA pair)
9
8
7
6
5
4
3
2
1
0
dsMock
dsFoxO
C
FB
Relative FoxO mRNA abundance
0.12
0.10
0.08
0.06
0.04
0.02
0
***
dsMock
dsFoxO
D
Relative Vg mRNA abundance
2000
1500
1000
500
0
***
dsMock
dsFoxO
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Oocyte length (mm)
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1.0
0.5
0
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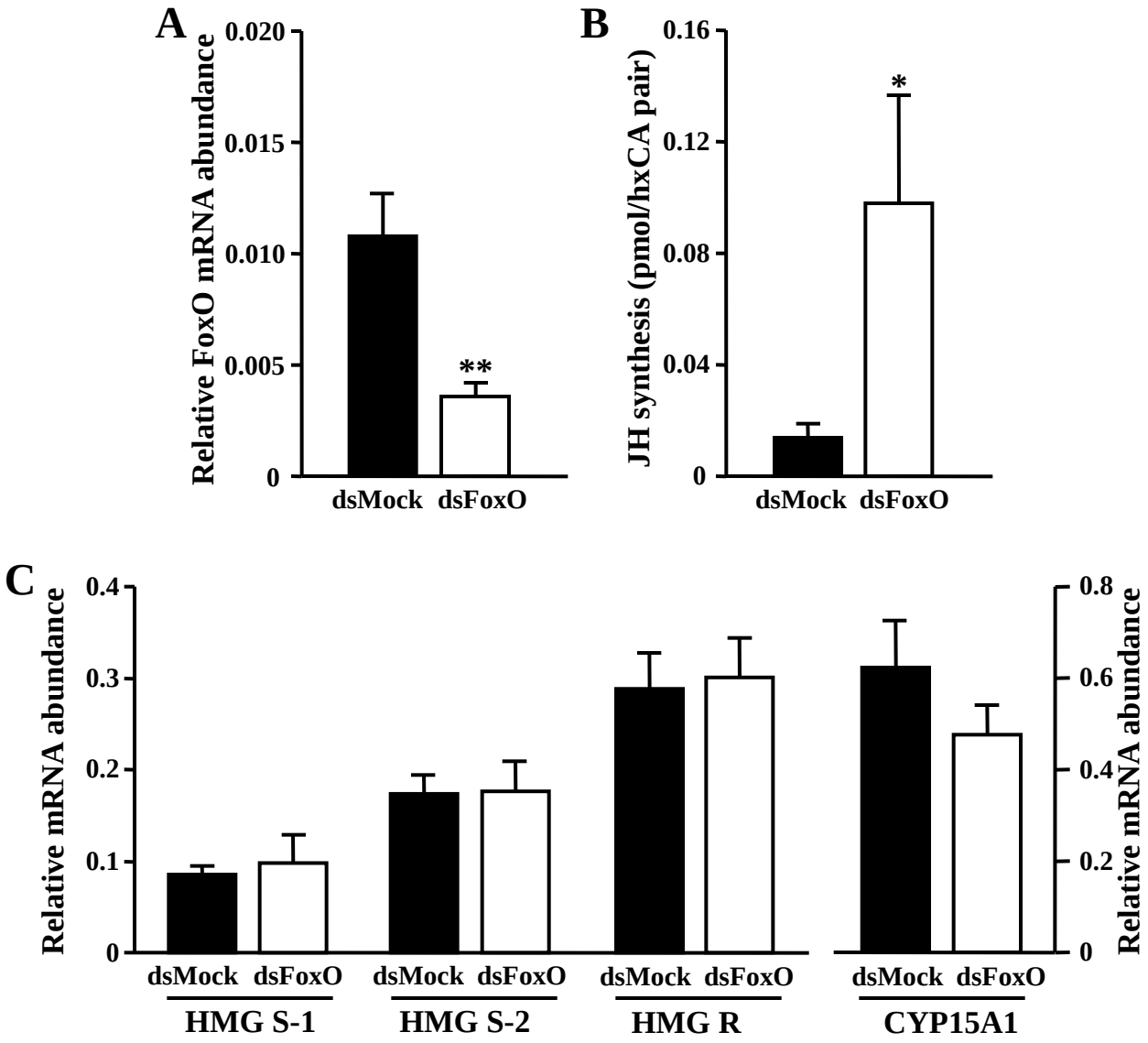
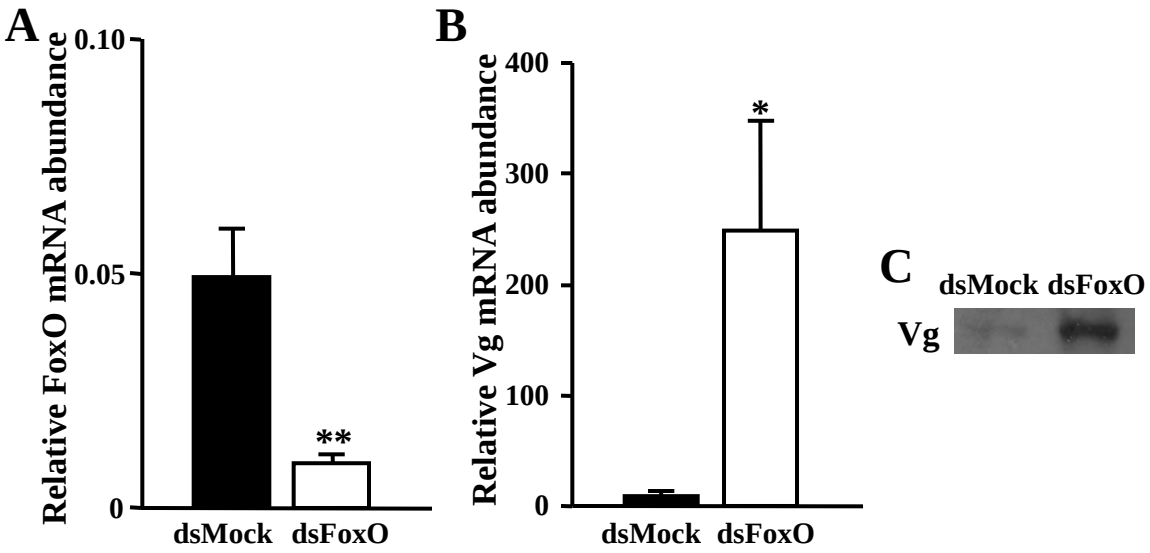
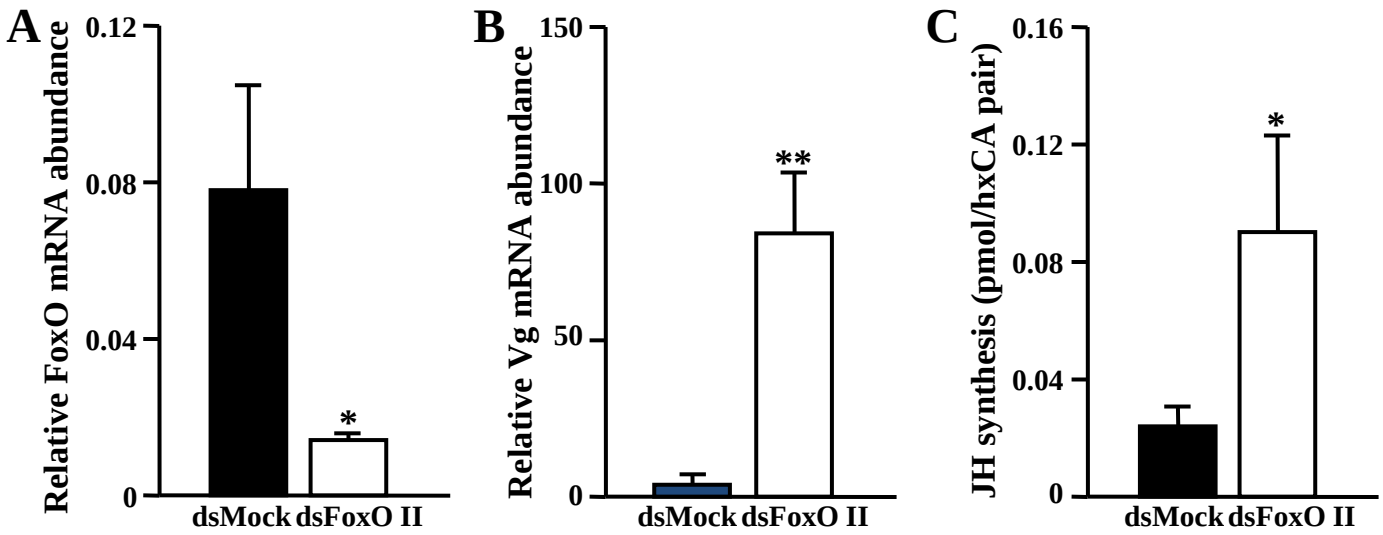
dsMock
dsFoxO

Figure(s)


A
Relative FoxO mRNA abundance
0.020
0.015
0.010
0.005
0
**
dsMock
dsFoxO
B
JH synthesis (pmol/hxCA pair)
0.16
0.12
0.08
0.04
0
*
dsMock
dsFoxO
C
Relative mRNA abundance
0.4
0.3
0.2
0.1
0
0.8
0.6
0.4
0.2
0
dsMock
dsFoxO
HMG S-1
HMG S-2
HMG R
CYP15A1

Figure(s)


A
Relative FoxO mRNA abundance
0.10
0.05
0
**
dsMock
dsFoxO
B
Relative Vg mRNA abundance
400
300
200
100
0
*
dsMock
dsFoxO
C
dsMock dsFoxO
Vg



Supplementary data

Fig. 1. Effect of BgFoxO RNAi (dsFoxO II) on starved *B. germanica* females. dsRNA targeting BgFoxO (*dsFoxO II*) or a non-specific dsRNA (*dsMock*) were administered in the first day of ootheca transport. The ootheca was removed 12 days later and dissections were performed at day 5 of the induced second gonadotrophic cycle. Animals received only water after the induction of the second cycle. (A) BgFoxO mRNA levels in fat body (n=5). (B) Vg mRNA levels (n=5). (C) Rates of JH synthesis by CA incubated *in vitro* (n=7-9). In (A) and (B) Y-axis indicates copies per copy of Actin 5C. Results expressed as the mean ± S.E. Asterisks represent significant differences (Student's *t* test, **P*<0.05; ***P*<0.005).

Table 1. Primer sequences.

Degenerated primers used for BgFoxO cloning

Forward	5′-AA(C/T)GCNTGGGGNAA(C/T)T-3′
Reverse-1	5′-TCNCC(C/T)TT(A/G)TC(C/T)TT(A/G)AA(A/G)TANGG-3′
Reverse-2	5′-ACCATCCA(C/T)TC(A/G)TA(A/G/T)AT(C/T)TG-3′

Primers used for synthesizing dsFoxO and dsFoxO II

Forward dsFoxO	5′-GTCCTATGCAGATCTCATCACGC-3′
Reverse dsFoxO	5′-CCATAGCCTCCAGTGTATGGTGA-3′
Forward dsFoxO II	5′-ACTCCTGGACGATCTTAACCTCAA-3′
Reverse dsFoxO II	5′-TTTGATAAACATCTTCTTCTATGTTTG-3′

Primers used for qPCR

Forward BgFoxO	5′-GAACTCAAGTCGCCGCAAC-3′
Reverse BgFoxO	5′-CGAGAGCGTGAGCCTCTTCT-3′
Forward Actin 5C	5′-AGCTTCCTGATGGTCAGGTGA-3′
Reverse Actin 5C	5′-TGTCGGCAATTCCAGGGTACATGGT-3′
Forward HMG-CoA synthase-1	5′-CTTCGCTTTACGGAGGTTTGGTC-3′
Reverse HMG-CoA synthase-1	5′-GCTGCGGCTTGATGTGCGAGAG-3′
Forward HMG-CoA synthase-2	5′-GCACAGACAGGGAGGACATCA-3′
Reverse HMG-CoA synthase-2	5′-CCTACTTCCAGCCATCCAATG-3′
Forward HMG-CoA reductase	5′-TTGTAGCTGATGGAATGACTCGT-3′
Reverse HMG-CoA reductase	5′-AGCAATGAAGCGTATAAACAAATG-3′
Forward CYP15A1	5′-ATGATGCGTTTGACGTGTGT-3′
Reverse CYP15A1	5′-GGAGACGCTGATCATCCAAT-3′
Forward Vg	5′-CTGGGCATTTGACAACACAACAT-3′
Reverse Vg	5′-TTGAAGAGCTGCTGGAGAGTTTG-3′